Thesis: Evaluation of Effects of Different Necrotic Enteritis Models in Chickens

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Abstract

The purpose of this research is to evaluate the effects of different necrotic enteritis (NE) models in boilers. Necrotic enteritis is a multifactorial acute enterotoxaemia that is often fatal and costs $6 billion annually around the globe. Currently, the most widely used research induction of NE is a combination of main causative agent *Clostridium perfringens* (CP) and *Eimeria maxima* (EM). Although occurrence of NE recently arose with CP toxin NetB without the presence of *Eimeria*. In this experiment, 9 sets of birds were given different treatments. All birds were treated with *Salmonella* Enteritidis, except negative control groups. Remaining groups received a combination of EM strains Guelph and M6 with different forms of CP, and different forms of CP alone to further explore the roles toxins and strains play in the pathogenesis of NE. Through the analysis of lesion scores, mortalities, body weights, and necropsies, all models were evaluated for severity of NE. Groups challenged with EM and CP Net B- (a dual infection model) gave the strongest indication of NE, with significantly higher lesion scores ($p<0.05$). Also showing signs of NE were the groups challenged by feed with CP, specifically the Net B+ form, though lesions scores were lower ($p<0.05$) than dual infection models. The unwashed form of CP, which contained toxins, metabolites, and by products from growth, showed the most drastic change in percentage bodyweight gain change ($p<0.05$). The washed form of CP, which consisted of the anaerobic bacteria alone, produced similar results, just slightly lesser change in percentage bodyweight gain. In conclusion CP alone did not create lesions, but did change performance characteristics which were typical of NE. These results provide insight regarding effective NE model, which will allow for preventative methods to be backed by research and science.

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Introduction

Necrotic enteritis (NE) is an acute enterotoxaemia which is considered the most common and financially devastating bacterial disease in modern broiler flocks (13). Additionally, NE causes flock depression, reduced performance, weight loss, secondary infections, morbidity, and mortality, ultimately leading to this financial loss. NE is multifactorial and can be influenced by several factors and causative agents. The primary causative agent is *Clostridium perfringens*, an anaerobic species which is normally found in the intestines of healthy chickens and turkeys (6). *Clostridium perfringens* causes NE when it begins producing toxins, as opposed to its non-toxin producing phenotype in a normal bird (14). *Clostridium perfringens* occurs in five different types, identified as A – E based on toxin genes, with types A and C the most prominent in poultry (6). The different types produce different toxins: alpha, beta, epsilon, iota, CPE, and NetB (6). The toxins produced by *Clostridium perfringens* are damaging to poultry, harming the small intestine, causing liver lesions, and in extreme cases, kills birds (6). The alpha-toxin has historically been considered the most important virulence factor, but in the early 2000's researchers showed that a novel toxin, NetB, can also play a major role in development of NE, especially in Europe and Australia (9).

Also involved in the prominence of NE is the presence of *Eimeria spp.* in the digestive system of chickens, especially *E. maxima* (5,14). Coccidiosis, the disease caused by *Eimeria*, is usually concurrent with other clinical signs of NE (5). *Eimeria* oocysts are nearly ubiquitous in poultry production systems; however, clinical disease occurs when chickens have ingested a high number of sporulated oocysts, and infection is characterized by a replication process that takes 4-7 days, dealing damage to the intestines (5). Recovered and infected birds both shed oocysts in feces, making coccidiosis difficult to control, which can be transmitted by direct contact, as well

as feces. Additionally, sporulated oocysts are incredibly hearty and can be difficult to rid barns of due to their resistance to common disinfectants (5). Because coccidiosis of the different portions of the digestive system occurs, it is relatively easy to distinguish species from each other purely based on location. For example, according to the Merck Vet Manual, *Eimeria maxima* will develop in the small intestine causing "dilatation and thickening of the wall; petechial hemorrhage; and a reddish, orange, or pink viscous mucous exudate and fluid." (5). This particular experiment utilized two strains of *Eimeria – E. maxima* M6 and *E. maxima* Guelph. The oocysts were provided by Dr. John Barta at the University of Guelph.

Other factors associated with the onset of NE are feed, environment conditions such as temperature, humidity, and pH, age of host, host genetics, concurrent disease, species of coccidia, and intestinal condition. NE is multi-factorial opportunistic disease, and can be explained only partially by science and research; however, we know some of the major factors contributing to onset. For example, damage to the mucosal layers of the intestine, shift of intestinal microflora, and rapid/drastic changes in dietary additives can lead to a higher rate of NE, which are often in conjunction with one or more of the other factors listed above (6). Also known to play a large role is the prevalence of animal byproducts in the broiler diet, such as fishmeal, as well as grains such as wheat and barley (6).

Generally, NE occurs worldwide, and is studied by labs across the country, as well as internationally. First discovered in 1961, NE was controlled by employing subtherapeutic antibiotics, often referred to as antibiotic growth promotors (AGPs) with some success (18). NE is defined by numerous studies done for dozens of years; however, the research is lacking in concrete and consistent results. It is imperative to strive for answers in terms of induction, and at length, produce solid preventatives and/or treatments.

Problem Justification

The main reason for studying NE is the financial damage it does to the poultry industry in the United States, as well as other countries. Although Necrotic Enteritis affects species outside of chickens, such as turkeys and quails, the focus of this experiment is on the effects of NE on chickens/the broiler industry. Globally, NE costs around $6 billion annually and occurrence is growing because the poultry industry is expanding each year (24). Worldwide, poultry is the 2nd most consumed meat, and is increasing in consumption every year. The Organization for Economic Co-operation and Development recently published 2017 per capita meat consumption for 63 countries and reported that poultry meat was the number one meat for 22 countries, as well as the highest consumed meat product as a total of all countries surveyed. In the United States, per capita poultry consumption was nearly double (48.9 kg/year) that of the next closest meat, beef and veal (25.9 kg/year) (11).

Domestically, the United States is the world's largest producer and second largest exporter of meat from chicken and turkey and is also a significant egg producer (23). It is crucial to better understand, prevent, and eventually eradicate necrotic enteritis. This study will shed light on the microbiology behind the occurrence of NE. In knowing the specific causes of NE, professionals can better combat and prevent NE worldwide.

Hypothesis/Objectives

The researchers hypothesize that subclinical NE will be reproduced in two different models. The first model hypothesized for induction of NE is a dual infection model of *Eimeria maxima* and *Clostridium perfringens* (NetB toxin negative). The second model hypothesized for induction of NE is a set of birds challenged with *Clostridium perfringens* (NetB toxin positive).

The researcher's hypotheses are based on prior research in history, recent research/breakthroughs, and models published by Wilson and co-authors (25).

In terms of historical research, the α-toxin of *Clostridium perfringens* was thought to be the main cause of NE (13). This toxin is an enzyme, phospholipase C, and is still relevant in current research; however, more recent research shows that a toxin, NetB can be a cause of NE (13). The most recent science can conclusively say that NE is multifactorial and involves a combination of causative agents and environmental factors. The first experiment in the three-experiment series revealed the science and results needed to construct a hypothesis for experiment 2. In conclusion to the first experiment, researchers successfully reproduced subclinical NE in a dual infection model of *Eimeria maxima* and a NetB negative *Clostridium perfringens*, as well as with a NetB positive *Clostridium perfringens* model.

The objective of this particular experiment is to establish reputable research through randomization and repetition of experiments, solidify current research about NE, and lay the groundwork for future research regarding treatment, prevention, and eradication of NE. More specifically, the objective is to gain a deeper understanding of the acute phase of NE. This involves looking at different parameters and data such as the environment, the different challenges, the physical results, and the statistical analyses. Another goal for this experiment is to move forward in the three-experiment manuscript to publish to the scientific community and have an impact on future experiments which will be able to utilize the results of this experiment.

Procedures/Methodology

Animals, Housing and Experimental Design

This experiment was conducted under approved animal care protocols from The Ohio State University Institutional Animal Care and Use Committee. In this experiment, Ross 708

broilers chicks were obtained from a local hatchery, neck tagged, and randomly assigned to floor pens with fresh pine shaving litter. Feed and water were provided *ad libitum*, except where indicated below, and ambient temperature was maintained at age-appropriate levels. During the first week, birds had 24h of light, followed by 1h of darkness, which was increased by 0.5h every other day until an 18h light : 6h dark pattern was reached. All birds were provided with basal starter (d0-14), grower (d14-28 or d14-35), and finisher (d28+ or d35+) based on NRC poultry requirements (10). For all experiments, body weight (**BW**) was measured the day of diseases induction, and then weekly following the NE disease period through the final day.

Bacterial Preparation

Salmonella enterica serovar Enteritidis (**SE**) was prepared and administered to all chicks on day of hatch, except for non-challenged control group (**NC**), as described by Shivaramaiah and co-authors (16, 17). The dose was retrospectively confirmed by plating ten-fold dilutions on trypic soy agar (**TSA**), as previously described and are reported in the data table (16, 21).

All CP isolates were acquired from clinical cases of NE, and species confirmation was determined by culture characteristics, Gram-stain, and PCR detection of select CP genes including alpha and NetB toxins (3, 12). To prepare inoculum, a single frozen aliquot of each strain was inoculated into individual tubes with tryptic soy broth (**TSB**) with 0.25% sodium thioglycolate (**TSBT)** and incubated in anaerobic jars at 37ºC for up to 24 h (1, 2, 20, 22). Cultures administered via oral gavage were washed by centrifugation and spectrophotometrically quantified (19). All CP CFU were retrospectively confirmed by plating ten-fold dilutions on TSA with sodium thioglycolate, and values are reported in appropriate data tables.

In-feed Administration of Clostridium

Treatment inoculum in all experiments were prepared by growing each strain independently in TSBT, with a fresh culture prepared each day. When CP was administered in the feed, CP was not washed by centrifugation, except where noted as "washed," similar to Jiang, et al. (7). To prepare supernatant treatment inoculum, once centrifuged, supernatant was collected. The remaining bacteria pellet was washed an additional two times to prepare inoculum for washed groups. Where combined strains of CP were administered in feed, equal amounts were mixed into a common container prior to washing by centrifugation. All CP treatments were diluted with TSBT and quantified spectrophotometrically (19, 20, 22). Each CP strain was administered in TSBT and mixed in the feed trough or placed on multiple cookie sheets in each treatment pen, as described below and in Table 1.

Table 1. Necrotic enteritis induction scheme. In this experiment, BW was measured on day 16, at the time of *Eimeria maxima* (EM) administration and on day 22 at the time of lesion score evaluation. All birds were inoculated with 1.2 x 10^4 CFU *Salmonella* Enteritidis on day of hatch. EM oocysts represent sporulated oocysts.
[1]CP1 administered by oral gavage at 2.0 x 10^8 CFU/chick, experiment 3
[2]EMG = *Eimeria maxima* Guelph strain; EMM6 = *Eimeria maxima* M6 strain
[3]CPB cultures were not washed by centrifugation unless indicated, reported as total CFU
[4]Combined with 1 kg of feed for administration to chickens, reported as total CFU

Exp.	Group	EM d16 (oocysts/bird)	CP1[1]	NetB CP in feed d17-d20	#/day CP in feed
3	NC				
	EMG	30,000	yes		
	EMM6	20,000	yes		
	CPB1 Low			100 mL, 1x10^{10} CFU	1
	CPB1 Unwashed			1 L, 1x10^{11} CFU[4]	2
	CPB1 Washed			1 L, 1x10^{11} CFU[4]	2
	CPB1 Supernatant			1 L supernatant from Washed	2

Experiment

A total of 350 day-of-hatch Ross 708 broiler cockerels were randomly assigned to treatment groups, each with n=40 unless otherwise specified. Groups included NC (n=70), EMG (n=60), EMM6 (n=60), low dose of CPB1 (**CPB1 Low**), high dose of unwashed CPB1 (**CPB1 Unwashed**), high dose of CPB1 supernatant (**CPB1 Supernatant**), or high dose of CPB1 washed (**CPB1 Washed**). The low dose of CPB1 was a 100mL volume of $1x10^8$ CFU/mL CP added to the feed once daily, while the high dose of CPB1 was a 1L volume of $1x10^8$ CFU/mL CP mixed into the feed twice a day, depicted in Table 1. Feed was removed up to six hours prior to challenge to encourage consumption of CP contaminated feed once it was distributed on multiple trays throughout each pen. The diet for all groups contained 1ppm of Diclazuril until 48 h prior to EM challenge on d16, at which point EMG and EMM6 were switched to non-medicated feed, in order to minimize the risk of coccidiosis before administration for experimental purposes. The chicks were challenged based on the schedule outlined in Table 1 below. On d22, BW was recorded for all birds, then half of the birds from each treatment group (20/treatment) were euthanized and lesion scores were measured based on a 0-4 scale according to Prescott, et al. (15, 25) (data not shown). Body weights were recorded weekly for the remaining 20 birds per pen through d56.

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Statistical Analysis of Data

The experiment was a completely randomized design which means that the treatments were assigned to experimental units (the birds) at random. The researchers chose a generalized linear model because it is assumed that Y (the body weight for each weigh day) is normally distributed and continuous, the errors were normally distributed and independent, while the X (treatment) is fixed, meaning the treatments never changed. Analysis of variance (ANOVA) is a statistical method used to test differences between two or more means. It initially told researchers if the model was different with a p-value, but it did not tell researchers what the treatment differences were in terms of each other. Therefore, a multiple comparisons test was chosen with Tukey's Honestly significant test. This told us if the treatment had a mean lower body weight at day x compared to EM +CP at a p value < 0.05. Tukey's is a more conservative test that is less likely to make a type I error (rejection). The p value tells researchers the likelihood of making a type I error, and the cut off used was 0.05 or 5%.

The lesion score data is ordinal, meaning that the values are discrete, and they go up in severity (diseases 0 healthy to 4 nearly dead). A mixed model was chosen because the scores given to each bird was independent and it was cited as an appropriate model to analyze if the treatment is associated with the mean score given among the 20 birds in each treatment. For body weight data, the data was continuous, meaning the numbers can go on forever; like height the value can be 5 ft 8 inches, but it can really be 5 ft 8.4395938394929...inches.

Statistical data was analyzed using JMP, a software program developed by SAS Institute Inc. JMP utilizes a graphical user interface and can be used on both Macs and Windows operating systems (8).

Replications Needed

This experiment is the second experiment in a three-part manuscript. The two replications of the first experiment will result in concrete science that can be applied to future experiments involving preventatives and/or treatments for NE.

Budget

This project was done under advisement from Dr. Lisa Bielke, a researcher from Ohio Agricultural Research and Development Center of the Animal Sciences department of Ohio State University. Most laboratory resources are available in Dr. Bielke's laboratory in Gerlaugh Hall, Wooster OH; however, expendable supplies were needed to complete this experiment.

Many of the expendable laboratory supplies used for this experiment including oocysts, protein extraction and purification reagents, biosafety supplies, animal housing and feed, and replacement costs of worn labware were covered through a SEEDS grant applied for in 2016. This funding request amounted to $2,250.

Findings

The researcher's findings indicated that two models successfully reproduced NE. Groups challenged with *Eimeria maxima* and *Clostridium perfringens* Net B-negative (a dual infection model) gave the strongest indication of necrotic enteritis presence, based on clinical signs and lesion scores. Clinical signs of NE include huddling of birds (for warmth), ruffled/missing feathers, and less consumption of food. The dual-infection model groups showed all of these signs, and in conjunction with lesion scores and percentage body weight gain decreased, these groups indicated induction of NE (13).

Also showing signs of NE, were the groups which were challenged by feed with *Clostridium perfringens,* specifically the Net B+ form. The unwashed form of *Clostridium perfringens* showed the most drastic change in percentage bodyweight gain change. The washed form of

Clostridium perfringens produced similar results with slightly lesser change in percentage bodyweight gain. In conclusion to the *Clostridium perfringens* results, *Clostridium perfringens* alone did not create lesions, but did change performance characteristics which are typical of NE. Graphical and statistical findings have been included below with captions interpreting the data.

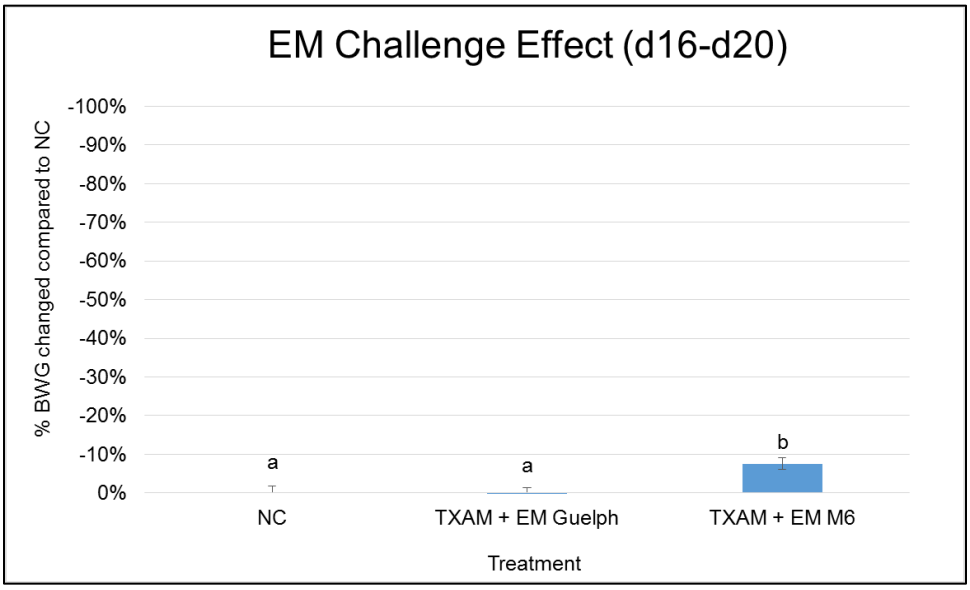

Figure 1, % Body Weight Gain (BWG) Change, *Eimeria* Challenge (M6/Guelph Compared to Negative Control) Shows that *Eimeria* Challenge alone did not a strong effect on BWG; however, the M6 strain of *Eimeria* caused a slight decrease in percentage body weight gained

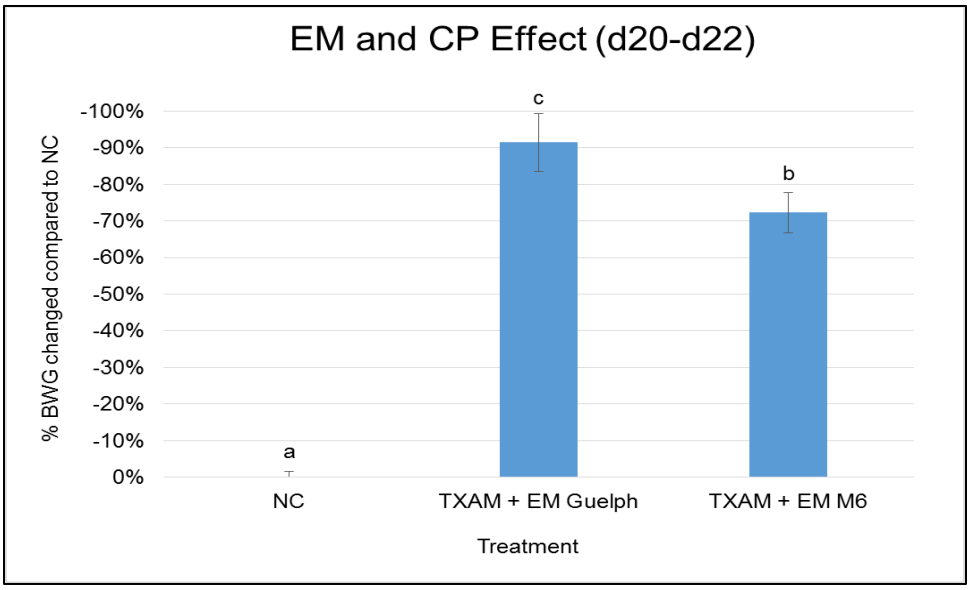

Figure 2, % Body Weight Gain Change, *Eimeria* + *Clostridium perfringens* (M6+CP/Guelph+ CP Compared to Negative Control) As compared to Figure 1, percentage body weight gain has drastically decreased with the dual infection models `

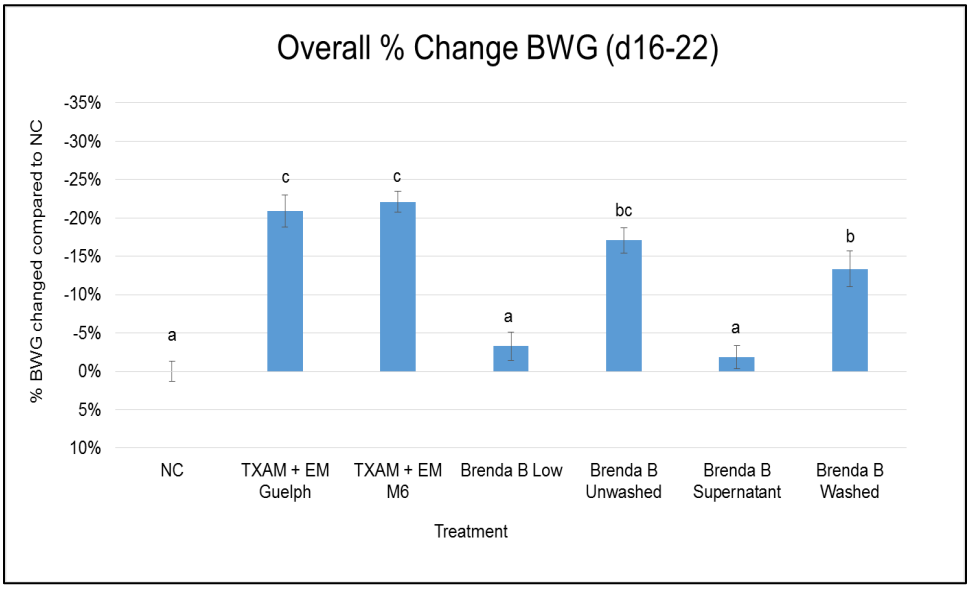

Figure 3, Overall % Change in Body Weight Gain (BWG) Compared to Negative Control Brenda B Low and Brenda B Supernatant did not result in significant percentage body weight gain decrease as compared to TXAM + EM Guelph/M6 and Brenda B Unwashed/Washed

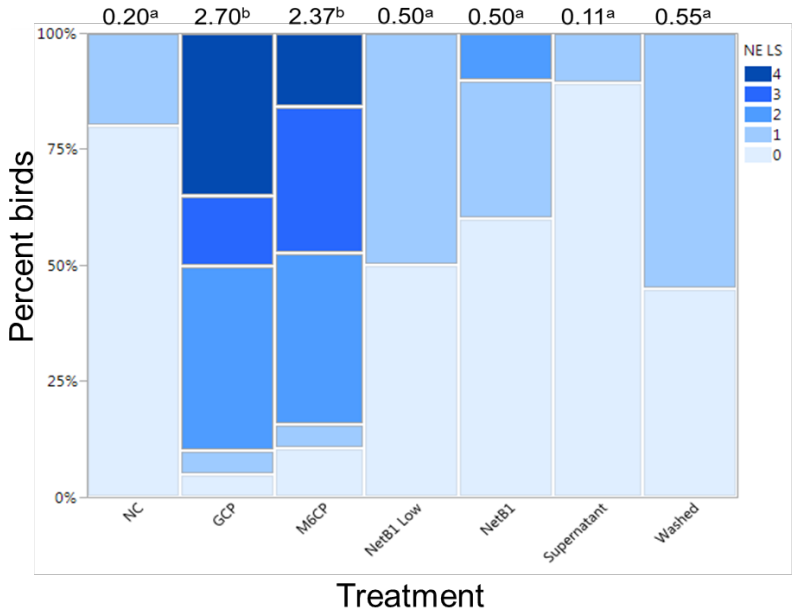

Figure 4, Mosaic Plot of Lesion Scores TXAM + Eimeria (M6 and Guelph) are the only groups which show lesion scores of 3 and 4. The plot shows that CP did not cause significant lesion scores, although Washed and Unwashed have ~50% of 1 scores and some with scores of 2 in the Unwashed group.

Tables 2 + 3, Overall Body Weight (BW) Body Weight Gained (BWG), % BWG and the % BWG changed relative to the Negative Control (% change BWG)

Group	d16 BW (g)	d22 BW (g)	Avg BWG	% BWG	% change BWG
NC	402.15 ± 8.91a	723.84 ± 16.48a	321.70 ± 9.37a	44.31 ± 0.60a	0.00 ± 1.35a
TXAM + EM Guelph	384.82 ± 9.24ab	594.54 ± 14.19de	209.72 ± 7.96cd	35.06 ± 0.92cd	-20.88 ± 2.07cd
TXAM + EM M6	347.38 ± 8.64b	530.64 ± 12.15e	183.26 ± 5.14d	34.52 ± 0.59d	-22.09 ± 1.34d
Brenda B Low	402.73 ± 10.68a	706.76 ± 18.09ab	304.03 ± 9.59a	42.86 ± 0.82a	-3.27 ± 1.85a
Brenda B Unwashed	396.28 ± 10.44a	630.36 ± 18.68cd	234.09 ± 9.62bc	36.75 ± 0.73bc	-17.08 ± 1.64bc
Brenda B Supernatent	390.14 ± 10.60a	690.63 ± 17.85abc	300.49 ± 9.13a	43.48 ± 0.66a	-1.87 ± 1.49a
Brenda B Washed	392.40 ± 9.06a	639.98 ± 14.73bcd	247.58 ± 9.92b	38.40 ± 1.03b	-13.35 ± 2.32b

		Eimeria maxima Challenge Effect			*Clostridium perfringens* Challenge Effect		
Group	d20 BW (g)	Avg BWG d16-d20 (g)	%BWG	% change BWG	Avg BWG d20-d22 (g)	%BWG	% change BWG
NC	611.50 ± 14.20a	291.35 ± 6.90a	34.07 ± 0.63a	0.00 ± 1.85a	112.34 ± 6.90a	15.56 ± 0.62a	0.00 ± 1.62a
TXAM + EM Guelph	586.67 ± 15.69b	201.85 ± 7.50b	34.15± 0.57b	0.23 ± 1.67a	7.87 ± 7.50c	1.33 ± 1.57b	- 91.50 ± 7.86c
TXAM + EM M6	508.97 ± 13.72c	161.59 ± 5.79c	31.51 ± 0.51c	-7.52± 1.51c	21.67 ± 5.79b	4.31 ± 0.86b	- 72.30 ± 5.50b

Summary

NE is a problem for the poultry industry, specifically in chickens due to its physical and financial effects. The harmful effects of the enterotoxaemia causes chickens to experience morbidity and death. Once NE is prevalent, it is difficult to dampen its effects without euthanizing and sterilizing barns with high-powered chemicals before introducing a new flock of

broilers. The financial effect of NE is currently felt for poultry producers as they deal with loss of flocks and large portions of income. Down the line, consumers may feel these effects as prices of meat are increased to make up for financial loss on the production side.

This research will help shed light on the processes of NE and allow for a more successful control/eradication of the occurrence of NE. In studying and experimenting with different models of NE, the researchers successfully induced NE in two models. In applying this research in conjunction with future research, industry professionals and veterinarians will be able to establish approved methods for operations to better handle NE.

Conclusions

In conclusion to this experiment, researchers can be confident in the induction methods found in two different experiments. After a third experiment, a manuscript can be published to display a further understanding of the induction of NE based on replications of experiments. This research will lead to preventative methods and treatment options down the road. Laying this ground level information and science is important to poultry operations now and will become increasingly important as researchers progress in terms of preventatives and treatments.

Current relevance to the industry is apparent, a result of knowing further microbiological components of NE. Environmental changes can be made to better promote healthy microflora in the gut. Researchers were able to see how NE was induced under certain environmental conditions, including temperature, bedding, pen size, pen occupants, and cleanliness of researchers and/or facility. Additionally, in knowing which of the causative agents are responsible for the induction of NE, efforts can be made to control these agents. While already well-studied, readers can be confident in methods to reduce occurrence of NE. One such method is to control coccidiosis in chickens is receiving coccidiostats early in development or via

vaccination to control later stages of *Eimeria* infection that will predispose flocks to NE outbreaks. Another method consists of having a well-balanced diet that avoids animal byproducts like fishmeal and grains like wheat and barley (6).

Future relevance can be considered the most important aspect of this research. Using this research, coupled with further research and experiments with a heavier focus on medicated feed/antibiotics, the most effective method of preventing and controlling Necrotic Enteritis can be employed. Also, the antibiotic free market is a rising area of concern, especially in Europe and the USA. Some U.S. companies are currently doing this, and it is vital that the companies and their faculty are presented with research and scientific proof as to why antibiotics are recommended and/or the right way to keep their chickens healthy and market-ready.

Recommendations

An interesting factor that didn't seem to catch much attention are the differences in the causative agents in various countries. Can the strains of *Eimeria* and *Clostridium* be isolated and listed for different countries and even regions within countries? My recommendations would be to compile lists of researched data to compile international databases of NE and its causes/isolated microbes.

In terms of recommendations for this particular research project, the researchers should progress this research further and establish preventative methods based on induction methods. Furthermore, this research could lead to prevention methods in different places in terms of climate, altitude, humidity, UV ray exposure, etc. to develop a concrete plan for future generations and healthier poultry productions.

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12. **PCR Detection of *Clostridium perfringens* Toxin Genes. Bacterial cultures with five replicates for each tested isolate were centrifuged for two minutes at room temperature at 14,000 RPM twice with sterile PBS to separate cells from the growth medium. After removing the supernatant, 400µL of cell lysis buffer (1M Tris-HCl, 500mM EDTA, 5M NaCl, 20% SDS) with 35µL of Proteinase K (10mg/mL) was added and incubated at 65°C for 20 minutes. For DNA purification, the pellet was washed with 200µL of 70% ethanol was and then TE (10mM Tris-HCl, 1mM EDTA) was added and samples were incubated in at 65°C one hour. DNA quantity and quality was measured with a**

NanoDrop, after which, samples were diluted in molecular grade water to a concentration of 75ng/µL and stored at -20°C until used.

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